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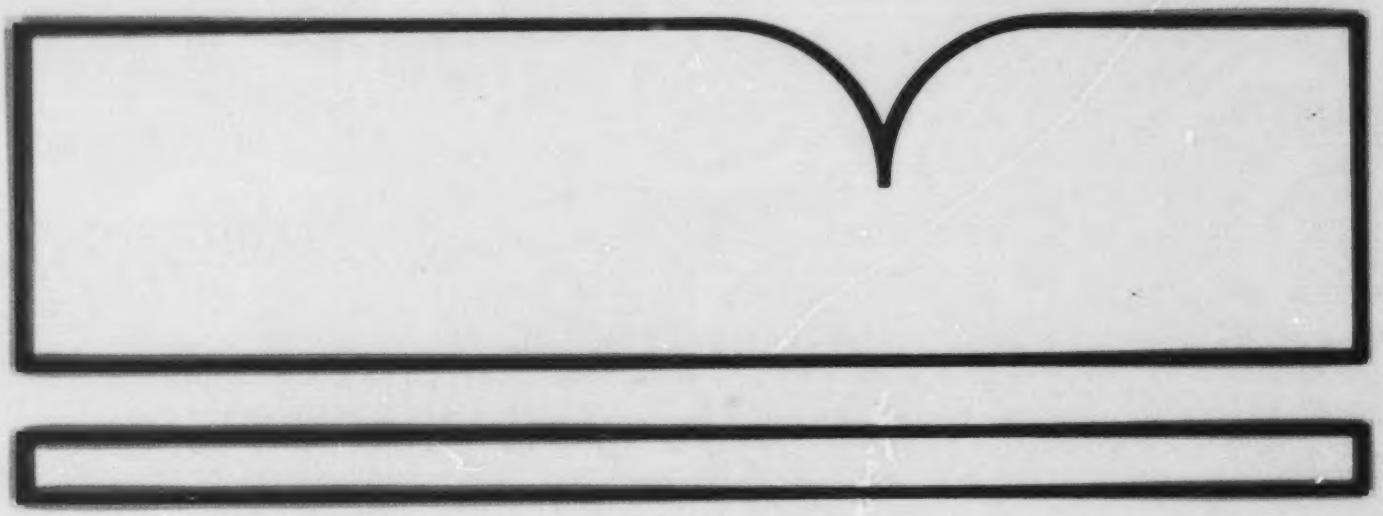

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ISOMER DEPENDENT BIOAVAILABILITY OF POLYCHLORINATED DIBENZO-P-DIOXINS AND DIBENZOFURANS FROM MUNICIPAL INCINERATOR FLY ASH TO CARP

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ABSTRACT

The isomer dependent bioavailability of PCDDs and PCDFs from municipal incinerator fly ash to freshwater fish was determined. It was observed that carp exposed to fly ash in a continuous flow exposure readily accumulated select isomers of PCDDs and PCDFs. A preference for greater retention of isomers substituted in the 2,3,7 and 8 positions was observed. The Bioavailability Index (ratio of contaminant level in fish to level in fly ash) decreased with increasing degree of chlorination.

INTRODUCTION

Previous studies by our laboratory have demonstrated that 2,3,7,8-TCDD will accumulate in freshwater fish directly exposed to municipal incinerator fly ash[1]. These studies showed that carp (Cyprinus carpio) exposed to two different municipal incinerator fly ash samples, each containing all 22 TCDD isomers, retain measureable concentrations of only 2,3,7,8-TCDD. The exposures appeared to follow a dose response relationship for bioaccumulation from each fly ash sample, however, the magnitude of bioaccumulation of 2,3,7,8-TCDD was not directly related to the concentration of 2,3,7,8-TCDD in fly ash for the two fly ash samples. The analysis of environmental samples by other groups[2] have shown that tetrachloro- to octachlorodibenzo-p-dioxins and dibenzofurans (PCDDs and PCDFs) can be found as residues in freshwater fish.

The objective of this study was to examine the bioavailability of tetrachloro- to heptachloro- CDDs and CDFs from municipal incinerator fly ash to freshwater fish. The municipal incinerator fly ash chosen for study was one that had been used in our previous work[1], and referred to as the "Midwest" fly ash. The test species chosen was carp for data comparability to previous studies. An important aspect of this study was the development of analytical calibration standards, and an evaluation of the analytical protocol which used isotope dilution mass spectrometry at each degree of chlorination to determine the contaminant level in biological tissue.

EXPERIMENTAL

Collection of Fly Ash

Fly ash was collected from electrostatic precipitators of a municipal incinerator and homogenized by tumbling in a large drum. The fly ash was then sized with a 400 mesh screen. The material which passed the screen was used for the exposure.

Fish Culture

Carp were obtained from the U.S. Fish and Wildlife Service, LaCrosse, Wisconsin, held at 20° C, and maintained on a #2 granule and trout pellet diet.

Exposure Conditions

The study consisted of a control and exposure aquarium each containing 40 L water and 30 carp (5-10 g). A flow of 80 L/day of 25 ± 1° C water was maintained throughout the fly ash exposure. The exposure aquarium was dosed with 1000 mg of fly ash in 250 mL water on a Monday, Wednesday, Friday basis. The aquarium was cleaned of excess food and fly ash before renewing the dose. The fish were fed daily 2% of their body weight. Fish were withdrawn from the aquaria for analysis at days 0, 30 and 60 and frozen whole. Duplicate samples of 3 fish each were collected on each sampling date.

ANALYTICAL PROCEDURES

Preparation of Fish Samples

Sample Extraction: Gill and digestive tract tissue were removed from each fish, and the fish body was rinsed with 50 mL 10% methanol/water. A composite of the three fish bodies were then ground in a stainless steel meat grinder. An aliquot (20 g) was blended with enough anhydrous sodium sulfate (Fisher Scientific) to dry the sample, transferred to a soxhlet extraction thimble, and spiked with 100 µl of Internal Standard Solution A. The sample was extracted with hexane/methylene chloride (Burdick and Jackson) (200 mL, 1:1) for 16 hours. A sample set consisted of a matrix blank (an uncontaminated sample), a reference contaminated fish sample and six fish samples from the study. After extraction, iso-octane (5 mL) was added to the extract and the sample was concentrated to 5 mL with a Kuderna-Danish Apparatus.

PCDD/PCDF Isolation: The extract was transferred to a 2.5 x 30 cm column containing (bottom to top) silica gel-60 (Merck-Darmstadt) (1 g), potassium silicate (5 g), sodium sulfate (5 g) and sulfuric acid (Baker Ultrex) (9 mL)/celite (Fisher Scientific) (20 g), and eluted with hexane (200 mL). The eluant was concentrated to 2.5 mL and transferred to a column containing alumina (1 g) (Fisher Scientific) in a disposable pipette. The column was eluted with carbon tetrachloride (Burdick and Jackson) (4 mL) and methylene chloride (6 mL). The methylene chloride fraction was transferred directly to a column

containing 350 mg of carbon (AMACO PX-21)/silica gel-60 (25 mg/g) packed into a disposable pipette. This column was eluted with an additional 10 mL of methylene chloride, and 20% benzene/methylene chloride (15 mL). The column was then eluted in reverse flow with toluene (Burdick and Jackson) (10 mL). The sample was spiked with $^{13}C_{12}$ 1,2,3,4-TCDD (500 pg), an internal standard used to normalize mass spectral response, and concentrated with a stream of dry filtered air to 0.5 mL, quantitatively transferred to a micro-vial, and further concentrated to 20 µL just prior to GC/MS analysis.

Preparation of Fly Ash Sample

Sample Extraction: Fly ash (5 g) was blended with glass beads (10 g, 400 mesh) and extracted for 24 hours with benzene in a Soxhlet extractor. The extract was concentrated to 250 µL using a Kuderna-Danish apparatus and a stream of dry filtered air. The extract was then diluted to 3 mL with iso-octane.

PCDD/PCDF Isolation: A 100 µL and a 20 µL aliquot of the extract were each spiked with 100 µL of Internal Standard Solution A, and processed through the alumina and carbon column steps described for the analysis of fish tissue.

GC/MS: GC/MS analyses were performed on both the fish samples and the fly ash using a Finnigan-MAT 8230 high resolution mass spectrometer. Two high resolution gas chromatograph columns were used, 30 m DB-5 and a 60 m SP-2330. The mass spectrometer was operated in an electron impact mode using multiple-ion selection (MIS) mass analysis. The internal standard $^{13}C_{12}$ 1,2,3,4,-TCDD was used to normalize mass spectral response; the relationship between $^{13}C_6$ 1,2,3,4-TCDD and $^{37}Cl_4$ 2,3,7,8-TCDD was used to evaluate mass resolution, and GC column performance[3]; method efficiency for each congener group was evaluated by determining the recovery of a stable isotope labeled isomer in each congener group vs. $^{13}C_{12}$ 1,2,3,4-TCDD; and natural isomers were quantified vs. the stable isotope labeled compound in each congener group. Natural 1,2,3,4-TCDD added to the sample at a level just above the target detection limit was used to establish the minimum level of detection in fish samples for which no TCDD was observed. A listing of the m/z values used for MIS is presented in Table 1. Ion ratios for natural analytes were within 10% of theoretical for qualitative identification.

Determination of Organic Carbon Content: The organic carbon content of the fly ash was determined gravimetrically following low temperature ashing.

Determination of Percent Lipid: The average percent lipid of the carp used for this study was determined on a random sampling of fish from the stock tank. Carp (8) were ground frozen and thoroughly mixed. A subsample (2 g) was blended with anhydrous sodium sulfate (10 g), packed into a 1 x 25 cm glass column and eluted with methylene chloride (25 mL) into a previously weighed aluminum pan. The solvent was evaporated to determine the lipid weight for calculating the percent lipid.

Table 1. High Resolution Ions Used for GC/MS Analysis of Natural and Stable Isotope Labeled PCDDs and PCDFs.

Data Acquisition: Multiple Ion Selection of the Following Ions:

Compounds	m/z Value
TCDF	303.9016, 305.8986
$^{13}C_{12}$-TCDF	317.9389
TCDD	319.8965, 321.8936
$^{37}Cl_4$-TCDD	327.8847
$^{13}C_6$-TCDD	327.9137
$^{13}C_{12}$-TCDD	333.9338
PCDF	339.8597, 341.8567
$^{13}C_{12}$-PCDF	351.9000
PCDD	355.8546, 357.8516
$^{13}C_{12}$-PCDD	367.8949
HxCDF	373.8207, 375.8178
$^{13}C_{12}$-HxCDF	385.8610
HxCDD	389.8156, 391.8127
$^{13}C_{12}$-HxCDD	401.8559
HpCDF	407.7817, 409.7788
HpCDD	423.7766, 425.7737
$^{13}C_{12}$-HpCDD	435.8169
OCDF	443.7398, 445.7369
OCDD	457.7377, 459.7348
$^{13}C_{12}$-OCDD	471.7750

Sample Introduction: Capillary Column directly inserted into the ionizer.
Ionization: Electron Impact, 70ev, 1mA emission current
Source Pressure: 5 x 10^{-6} torr.
Ionizer Temperature: 250° C.
Mass Resolution: 5000, 10% valley
Scan Rate: 15 MIS cycles per 10 sec.
GC Column: 30 m DB-5, 30 m SP2340
Linear Velocity: 40 cm/sec Helium
Temperature Program: 80° C (2 min), 4°/min to 250° (30 min)

Validation of Analytical Methodology

Analytical Standards: A set of analytical standards for the calibration of the mass spectrometer and the internal standard spiking solution of stable isotope labeled compounds needed to be developed before analysis of samples could begin. Because sufficient quantities of each chemical were not available to weigh, standard solutions had to be purchased (Cambridge Isotope) and diluted to proper concentrations. This was accomplished in the following manner:

First, the mean mass spectral response for the molecular ion for three weighed solutions of equal concentration of each 1,2,3,4-TCDD and octachlorodibenzo-p-dioxin (OCDD) was determined. Next, dilutions of penta-, hexa-, and heptachloro- CDDs were made and their mass spectral response was measured on a linear extrapolation between the response for TCDD and OCDD. Dilutions of the $^{13}C_{12}$ labeled materials were then made until their mass spectral responses equaled that of the natural analog for the same ions, i.e., $m^{\cdot+}$, $(m+2)^{\cdot+}$. Concentrations of stock solutions for $^{37}Cl_4$ 2,3,7,8-TCDD and 2,3,7,8-TCDD had previously been established, and found to be within 5% of their expected response as compared to the PCDD response curve. The mean mass spectral response for three weighed solutions of OCDF was

approximately 2X greater than for an equal concentration of OCDD. Subsequently, mass spectral responses of the remaining labeled and natural PCDFs from solutions of equal concentration to PCDDs of the same degree of chlorination, were also higher by approximately a factor of two. Therefore, no further adjusting of the concentration of these solutions was done before calibration and spiking standards were prepared.

Calibration Standards: Five calibration standards were prepared for GC/MS analyses. Each standard contained 100 pg/μL $^{13}C_{12}$ 1,2,3,4,-TCDD for normalizing each GC/MS analyses. Standards A, B and C each contain the labeled compounds at a concentration equivalent to 50% recovery while standards D and E were equivalent to 100% recovery. The concentration of natural isomers increased in each standard from A to E. The range of concentrations was 2.5 to 100 pg/μL for TCDD and TCDF; 5 to 200 pg/μL for PeCDD and PeCDF; 10 to 400 pg/μL for HxCDD, HxCDF, HpCDD and HpCDF; and 20 to 800 pg/μL for OCDD and OCDF.

Internal Standard Solution A: A solution of stable isotope labeled compounds is described in Table 2. Each sample was spiked with 100 μL of this solution.

Table 2. Concentration of Standards in Internal Standard Spiking Solution A.

Compound	Concentration in Solution pg/μl	Concentration in 20 g Tissue ppt
$^{13}C_{12}$-2,3,7,8-TCDD	5	25
$^{13}C_{6}$-1,2,3,4-TCDD	5	25
$^{13}C_{12}$-2,3,7,8-TCDF	5	25
$^{13}C_{12}$-1,2,3,7,8-PCDD	10	50
$^{13}C_{12}$-1,2,3,7,8-PCDF	10	50
$^{13}C_{12}$-1,2,3,6,7,8-HxCDD	20	100
$^{13}C_{12}$-1,2,3,4,7,8-HxCDF	20	100
$^{13}C_{12}$-1,2,3,4,6,7,8-HpCDD	20	100
$^{13}C_{12}$-OCDD	40	200
Natural 1,2,3,4-TCDD	1	5

Method Efficiency and Precision of Data

Fish: In an effort to evaluate method efficiency for PCDDs and PCDF, and evaluate the precision of quantification, replicate analysis (N = 5) of a reference fish sample previously used at ERL-Duluth for the U.S. EPA National Dioxin Study was completed. Results of these analyses are shown in Table 3. Although the method efficiency for hexachloro- and heptachloro- compounds was below the generally accepted value of 50%, the precision of quantification of the natural analyte was within the quality assurance requirement of ±50% for low part-per-trillion level determinations set by the National Dioxin Study[4].

Fly Ash: Method efficiency for the two aliquots of fly ash extract exceeded 50% for each stable isotope labeled analog. It was recognized that the extraction technique used for the fly ash may not be exhaustive. therefore, no evaluation of accuracy or precision was attempted.

Table 3. Results for GC/MS Analysis of Reference Fish Tissue for Tetrachloro- to Heptachloro- DDs and DFs.

Compound	Level of Spike pg/g	Method Efficiency Mean*	Range	Quantification Mean*	Range
$^{13}C_{12}$2,3,7,8-TCDD	25	58	36-72	55(1)	47-59
$^{13}C_{12}$2,3,7,9-PeCDD	50	46	33-60	3.4(1)	3-4
$^{13}C_{12}$2,3,6,7,8-HxCDD	100	40	32-52	7.4(1)	6-9
$^{13}C_{12}$2,3,4,6,7,8-HpCDD	100	29	18-48	12(1)	11-13
$^{13}C_{12}$2,3,7,8-TCDF	25	55	35-69	14(1)	14-15
$^{13}C_{12}$2,3,7,8-PeCDF	50	50	31-70	1(.5)	1.0-1
$^{13}C_{12}$2,3,4,7,8-HxCDF	100	40	32-57	2.5	2.0-3

*N = 5

RESULTS

GC/MS results indicate that a series of tetrachloro- to heptachloro- DDs and DFs will bioaccumulate in freshwater fish when exposed to municipal incinerator fly ash. Table 4 provides quantitative data for the levels of PCDDs and PCDFs, for which ERL-Duluth has analytical standards, in fish from days 0, 30 and 60 of the exposure. As observed in our previous studies of TCDDs, a unique selectivity was observed for the bioavailability of isomers substituted in the 2,3,7, and 8 positions. Figure 1 graphically demonstrates the relationship between PCDD and PCDF isomers in fly ash and those accumulated and/or retained in fish. For example, the ratio of 1,2,3,4,6,7,9-HpCDD to 1,2,3,4,6,7,8-HpCDD in the fly ash is approximately 1.5:1, while this ratio has changed to 1:50 in fish exposed to the fly ash for 60 days. Although a complete set of analytical standards is not available at ERL-Duluth, peak assignments based upon literature[5,6] data and limited standards available at ERL-Duluth indicate that the predominant isomer in the fish tissue for TCDDs is 2,3,7,8; for TCDFs is 2,3,7,8; for PeCDDs is 1,2,3,7,8; and for PeCDFs is 2,3,4,7,8. We also observed three hexachloro- CDDs, and four hexachloro- CDFs with the 2,3,7 and 8 positions filled, 1,2,3,4,7,8-, 1,2,3,6,7,8-, and 1,2,3,7,8,9-; and 1,2,3,4,7,8- (not resolved from 1,2,3,4,7,9-), 1,2,3,6,7,8-, 1,2,3,7,8,9-, and 2,3,4,6,7,8-, respectively. The exact reasons for the observed selectivity are not known. None of the analytes presented in Table 4 were measured above the minimum level of detection in the 60 day control fish sample. Future experiments at ERL-Duluth will be designed to help understand these observations.

Table 4. Results of GC/MS Analysis of Exposure Fish at Days 0, 30 and 60 for Select PCDDs and PCDFs.

	Concentration of PCDD/PCDF pg/g (d.l.) "Day of Exposure"		
Compound	0	30	60
2,3,7,8-TCDD	ND(1)	3.6	7.4
1,2,3,7,8-PeCDD	ND(1)	70	43
1,2,3,6,7,8-HxCDD	ND(1)	47	105
1,2,3,4,6,7,8-HpCDD	ND(1)	63	104
2,3,7,8-TCDF	ND(1)	3.5	7.6
2,3,4,7,8-PeCDF	ND(1)	10	14
1,2,3,4,7,8-HxCDF 1,2,3,4,7,9-HxCDF	ND(1)	11	23
1,2,3,4,6,7,8-HpCDF	ND(1)	20	27

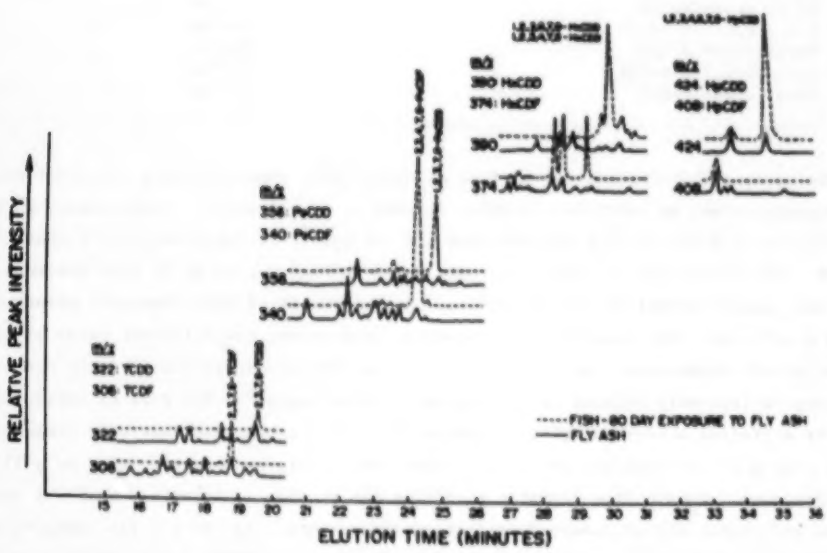

Figure 1. Qualitative Mass Chromatogram Comparison of PCDD and PCDF Isomers in Fly Ash and Carp exposed to Fly Ash for 60 Days.

The same mass spectral parameters were used to analyze both the fly ash and the fish. Although a potential interference could arise between the M+4 ion of the $^{13}C_{12}$ labeled PCDF and the natural PCDD quantification ion, M+2, the concentration of a PCDD isomer in fly ash was generally 10x greater than the concentration labeled PCDF resulting in less than a 5% potential error for co-eluting compounds. It also was noted that the labeled PCDF did not gas chromatographically co-elute with the PCDDS accumulated in fish. Results of fly ash analysis are presented in Table 5. The concentration of isomers for which no analytical standard was available are based upon the assumption that each compound in a congener group will have an equal mass spectral response to a known standard compound[7].

Table 5. Results for GC/MS Analysis of Municipal Incinerator Fly Ash for PCDDs and PCDFs.

Compound	Concentration on Fly Ash (<400 mesh) pg/g
2,3,7,8-TCDD	1,260
Total other TCDD	68,240
2,3,7,8-TCDF	1,660
Total other TCDF	41,110
1,2,3,7,8-PeCDD	12,460
Total other PeCDD	227,560
2,3,4,7,8-PeCDF	7,310
Total other PeCDF	75,960
1,2,3,6,7,8- and 1,2,3,4,7,8-HxCDD	50,540
Total other HxCDD	326,900
1,2,3,4,7,8-HxCDF	8,190
Total other HxCDF	70,720
1,2,3,4,6,7,8-HpCDD	140,980
Total other HpCDD	165,070
1,2,3,4,6,7,8-HpCDF	36,940
Total other HpCDF	16,620

The apparent bioavailability to fish of hydrophobic organochlorine compounds associated with organic carbon on particles in water depends on many factors. Measurements of bioaccumulation of PCDD and PCDF isomers from fly ash should be interpreted with these factors in mind. Concentrations of PCDDs and PCDFs found in fish following 60 days exposure to relatively small amounts of fly ash reflect the net result of many transport phenomena, each having a different rate constant. For example, each isomer has different rates of uptake, metabolism and depuration. We have found that the rate of depuration of PCDDs and PCDFs from carp is inversely related to the degree of chlorination[8]. The rate of uptake from water by diffusion across the gills, however, is likely to be also inversely proportional to the degree of chlorination due to the effect of increasing molecular size on gill membrane diffusion rate. Uptake from ingested particles can provide an important route of uptake with an additional set of isomer-dependent rate constants. In the fly ash exposure reported here, it is unlikely that water concentrations of PCDDs and PCDFs due to desorption from fly ash could result in the observed fish concentrations. Thus, ingestion of fly ash by carp, as in the case of sediment exposures, probably is the most important route of uptake.

In order to compare the isomer-dependent bioavailability of PCDDs and PCDFs to fish in fly ash, we calculated a Bioavailability Index (BI) for isomers identified in fish. The BI

is defined as the ratio of the concentration of chemical accumulated in 1 g of fish lipid to the concentration in 1 g of carbon on the fly ash (or other contaminated particulate). These ratios were normalized by setting the value to 1.0 for 2,3,7,8-TCDD and are reported in Table 6 for fish from the 60 day exposure to fly ash. The BIs for tetra- to heptachloro- DDs decrease from 1.00 to 0.11 while the values for DFs decreased from 0.77 to 0.12. The decrease in BI parallels increasing molecular weight[1], decreasing water solubility and increasing Kow. The increased binding affinity of the higher molecular weight compounds to the fly ash carbon may result in decreased bioavailability. The BIs calculated in this study only indicate trends between congeners. It is expected that the BIs will differ for other fly ash samples with a range of physical and chemical characteristics. More studies will have to be conducted in order to evaluate the factors which control differences in bioavailability.

Table 6. Comparative Results of the Level of PCDDs and PCDFs in Fish and Fly Ash for Select PCDDs and PCDFs, and the Bioavailability Index for this Particular Exposure.

Compound	Concentration Fish pg/g lipid	Concentration Fly Ash pg/g Carbon	BI	BI Normalized to TCDD
2,3,7,8-TCDD	93	1.26×10^5	7.4×10^{-4}	1.00
2,3,7,8-TCDF	95	1.66×10^5	5.7×10^{-4}	.77
1,2,3,7,8-PeCDD	540	1.25×10^6	4.3×10^{-4}	.58
2,3,4,7,8-PeCDF	180	7.31×10^5	2.5×10^{-4}	.34
1,2,3,6,7,8-HxCDD	1300	5.05×10^6	2.6×10^{-4}	.35
1,2,3,6,7,8-HxCDF	290	8.19×10^5	3.5×10^{-4}	.47
1,2,3,4,6,7,8-HpCDD	1300	1.65×10^7	7.8×10^{-5}	.11
1,2,3,4,6,7,8-; 1,2,3,4,7,9-HpCDF	340	3.69×10^6	9.2×10^{-5}	.12

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